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(54) Method of detecting DAF molecules in feces

Methode zum Nachweis von DAF-Molekülen im Stuhl

Méthode de décèlement de molécules de DAF dans les selles

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WO-A-86/07062 **JP-A- 6 317 588**

- **CHEMICAL ABSTRACTS, vol. 120, no. 15, April 11, 1994 Columbus, Ohio, USA T. HARA et al. "A monoclonal antibody against human decay-accelerating factor (DAF, CD55), D17, which lacks reactivity with semen-DAF", page 807, no. 189 246v; & Immunol. Lett. 1993, 37(2-3), 145-52.**
- **CHEMICAL ABSTRACTS, vol. 106, no. 23, June 8, 1987 Columbus, Ohio, USA M.A. DAVITZ et al. "Isolation of decay accelerating factor (DAF) by a two-step procedure and determination of its N-terminal sequence" page 552, no. 194 206x; & Immunol. Methods 1987, 97(1), 71-6**
- **Koretz et al, Br. J. Cancer, 1992, vol. 66: 810-814.**

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Description

[0001] The present invention relates to a method of detecting colorectal cancer.

[0002] In general medical examination, it is conventional practice to detect occult blood in faeces for detecting colorectal cancer. When occult blood is detected in faeces, the photographic examination based on a contrast medium used in the large intestine and the colorectal endoscopic examination of the colon are carried out to detect colorectal cancer. However, occult blood is not always found in faeces from the colorectal cancer patients. For this reason, when the examination is based on the detection of occult blood in faeces alone, colorectal cancer is sometimes not detected.

[0003] WO-A-86/07062 describes a method of making anti-DAF (decay accelerating factor) monoclonal antibodies and their use in detecting DAF in blood cells, spermatozoa and seminal plasma. Chemical Abstracts, vol. 120, no.15, pg 807,189246v,1994 describes a monoclonal antibody against human decay accelerating factor which lacks reactivity with semen DAF. Chemical Abstracts, vol 106, no. 23, pg 552,194206x, 1987 describes the isolation of DAF and determination of its N-terminal sequence.

[0004] It has been recently immunologically found that the expression of DAF molecules is enhanced in a colorectal cancer tissue. DAF molecules, i.e., decay accelerating factor, are glycoproteins which regulate the activation of the autologous complement cascade by promoting the catabolism of C3 and C5 convertases. It has been revealed that almost no DAF molecules are expressed in a normal epithelial cell on colorectal mucosa, but that DAF molecules are intensely expressed, particularly, on an apical surface of a cancer gland duct of a colorectal cancer cell. Koretz *et al.*, Br. J. Cancer (1992) vol. 66:810-814 is concerned with DAF in normal colorectal mucosa, adenomas and carcinomas.

[0005] The present inventors have assumed that faeces from colorectal cancer patients show an increased amount of DAF molecules.

[0006] It is an object of the present invention to provide a method of easily detecting the occurrence of colorectal cancer.

[0007] It is another object of the present invention to provide a method of detecting colorectal cancer by measuring DAF molecules which are synthesized by colorectal cancer cells and present in faeces.

[0008] It is further another object of the present invention to provide a method of detecting colorectal cancer in the absence of occult blood, by measuring DAF molecules which are synthesized by colorectal cancer cells and present in faeces.

[0009] According to the present invention, there is provided a method of detecting colorectal cancer in a patient, which method comprises:

- reacting an anti-DAF (decay accelerating factor) antibody with a supernatant of a solution of faeces from the patient;
- measuring an amount of the antibody which has bonded to DAF molecules by an antigen-antibody reaction with the DAF molecules in the supernatant; and
- detecting thereby if the patient has colorectal cancer

[0010] A solid phase anti-DAF antibody can be reacted with the supernatant of a faecal solution and then the resultant reaction product is reacted with a labelled anti-DAF antibody or fragment thereof to measure the amount of the bonded DAF molecules.

[0011] A solid phase anti-DAF antibody may be reacted with the supernatant of a faecal solution and then with an anti-DAF antibody and then the resultant reaction product is reacted with a labelled immunoglobulin or fragment thereof to measure the amount of the bonded second anti-DAF antibodies. The immunoglobulin can be a monoclonal immunoglobulin or a polyclonal immunoglobulin. The label is generally an enzyme label or a radioactive substance label.

[0012] The invention also proves use of an anti-DAF antibody in a method of detection of colorectal cancer by detecting the presence/of DAF molecules in faeces.

[0013] In the accompanying drawings:

[0014] Fig.1 is a graph showing DAF concentrations in faeces from colorectal cancer patients and a control group of patients having no colorectal cancer.

[0015] Fig.2 is a graph showing changes of DAF concentrations in faeces from pre-operation of colorectal cancer to post-operation.

[0016] The detection method of the present invention will be explained in detail hereinafter.

Preparation of supernatant of faecal solution

[0017] A predetermined amount of faeces are mixed with a buffer solution, the mixture is stirred, and then a supernatant is separated by centrifugation.

Preparation of anti-DAF antibody

[0018] Human DAF molecules are purified from erythrocyte membrane to prepare anti-DAF monoclonal antibody and anti-DAF polyclonal antibody.

Measurement of DAF molecules in feces

[0019]

1) The anti-DAF antibody may be an anti-DAF monoclonal antibody or anti-DAF polyclonal antibody. The anti-DAF antibody can be converted to a solid phase, on a microtiter plate or beads, and then reacted with the supernatant of the faecal solution.
2) After the above reaction product is washed, an enzyme- or radioactive substance-labeled anti-DAF monoclonal antibody or anti-DAF polyclonal antibody is reacted with the washed reaction product to measure an amount of the bonded DAF molecules.

[0020] Otherwise,
2') After the above reaction product is washed, an. anti-DAF monoclonal antibody or an anti-DAF polyclonal antibody is reacted with the reaction product. The resultant reaction product is washed, and then reacted with an enzyme- or radioactive substance-labeled antimonoclonal immunoglobulin antibody or antipolyclonal immunoglobulin antibody to measure the bonded second anti-DAF antibodies.

Preparation of calibration curve

[0021] A calibration curve is prepared on the basis of purified DAF in a known amount, and the DAF amount in a sample is calculated on the basis of the calibration curve and expressed as an amount in 1 g of faeces.
[0022] According to the method of the present invention, an amount of at least 0.4 ng in 1 g of faeces can be accurately measured.
[0023] DAF in faeces from a colorectal cancer patient, detected by the method of the present invention, is assumed to be derived from cancer cells or erythrocyte in blood from colorectal cancer. Since, however, DAF is detected even in faeces from a colorectal cancer patient who is negative with regard to occult blood, it is assumed that above detected DAF includes DAF in faeces, derived from cancer cells. DAF in faeces from a group of patients having no colorectal diseases (to be referred to as "control group" hereinafter) is less than the detectable sensitivity, and it is therefore assumed that normal colorectal mucosa secretes no DAF.
[0024] Colorectal cancer screening based on occult blood in faeces has a problem of false negative results because that colorectal cancer does not cause detectable bleeding in some cases. The method of the present invention is advantageous in that colorectal cancer which causes no detectable bleeding can be sometimes detected by detecting DAF in faeces.
[0025] The present invention will be explained more in detail hereinafter with reference to Examples, in which "%" stands for "% by weight" unless otherwise specified.

Example and Comparative Example

Preparation of supernatant of faecal solution

[0026] Faeces in an amount of 1 to 5 g were mixed with a buffer solution in the same amount as that of the faeces, and the mixture was stirred. The buffer solution was a sodium phosphate buffer solution containing 1 % of bovine serum albumin, 0.05 % of Tween (Trademark) and 1 mM of phenylmethylsulfonyl fluoride. The stirred mixture was centrifugally separated for 15 minutes at 20,000 g. The supernatant was sampled and freeze-stored at -80°C before the measurement.

Preparation of anti-DAF mouse monoclonal antibody

1) Purification of human DAF molecules

[0027] Erythrocyte was separated from 50 ml of human blood, and 400 ml of a 5 mM sodium phosphate buffer solution containing 1 mM of EDTA and 0.1 mM of phenylmethylsulfonyl fluoride was added to the separated erythrocyte. The mixture was stirred and centrifugally separated at 48,000 g, and a precipitate of an erythrocyte substrate was collected.

[0028] The above erythrocyte substrate in an amount of 1 g was diluted to 5 mg/ml with a 5 mM sodium phosphate buffer solution, and 80 ml of butanol was added. The mixture was stirred and then centrifugally separated at 12,000 g, and an aqueous layer between an upper layer of butanol and a lower layer of a precipitate was recovered. Then, the aqueous layer was subjected to a DEAE-Sephacel (Trademark) ion exchange column, and a crude fraction of DAF molecules was separated with a 0.02 M tris buffer solution containing 0.04 M of sodium hydrochloride and 0.1 % of Nonidet P-40 (Trademark; NP-40).

[0029] Further, the crude fraction of DAF was subjected to a phenyl-Sepharose (Trademark) column, and the DAF was separated with a 0.04 M sodium phosphate buffer solution containing 0.3 M of sodium hydrochloride and 0.1 % of NP-40 and then with a 0.05 M sodium phosphate buffer solution containing 0.05 M of sodium hydrochloride and 1 % of NP-40. Further, the DAF was separated through a hydroxyapatite column.

2) Preparation of anti-DAF mouse monoclonal antibody

[0030] A BALB/C mouse was immunized with a suspension of 5 μg of the purified DAF in a Freund adjuvant containing dead tuberculosis bacillus twice at an interval of one week. After three weeks, spleen cells were taken out and fused with NS-1 mouse myeloma cells using polyethylene glycol. The supernatant of a culture of the so-prepared hybridoma was reacted with a nitrocellulose membrane blotted with the purified DAF, and screened by detecting bonded mouse monoclonal antibody with an ABC kit (Trademark) supplied by Vector Laboratories, to give hybridomas which produced four kinds of anti-DAF monoclonal antibodies.

[0031] All of the produced monoclonal antibodies were IgG1 kappa. Of these four kinds of monoclonal antibodies, 1C6 monoclonal antibody which recognized an active portion of DAF molecules was used for the detection of stool DAF. Hybridoma which produced 1C6 monoclonal antibody was intraperitoneally injected into a mouse to prepare ascites, which was sampled.

[0032] 1C6 anti-DAF monoclonal antibody IgG was separated and purified from the ascites by an ammonium sulfate precipitation method and DEAE chromatography (using "Toyopearl" (Trademark) supplied by Tosoh Corp.).

Preparation of rabbit anti-DAF polyclonal antibody

[0033] The purified DAF was infused into 10 ml of rabbit erythrocyte by incubating it at 37°C for 1 hour. This erythrocyte was washed and dissolved, and cytoplasmic membrane was centrifugally separated. The cytoplasmic membrane was suspended in a Freund adjuvant containing dead tuberculosis bacillus, and the rabbit from which the erythrocyte had been separated was immunized with the so-prepared suspension. Blood was taken from the immunized rabbit, serum was separated, and rabbit IgG was separated and purified by an ammonium sulfate precipitation method and DEAE chromatography.

[0034] The specificity of the above-obtained antibody was examined by an immunoblotting method. That is, a crude extract of human erythrocyte substrate was electrophoresed in SDS polyacrylamide gel, blotted into a nitrocellulose membrane, and reacted with the rabbit DAF polyclonal antibody. When bonded rabbit IgG antibody was detected with a Vectastain (Trademark) ABC kit (Vector Laboratories, Inc., CA), a single band was detected in a site corresponding to the molecular weight of 70 kD of the DAF molecules, so that the specificity of the rabbit anti-polyclonal antibody was confirmed.

Measurement of DAF molecules in faeces

[0035] The 1C6 DAF mouse monoclonal antibody having a concentration of 2 μg/ml was placed in wells of a microtiter plate in an amount of 100 μl per well. After a reaction at 4°C for 12 hours, a sodium phosphate buffer solution containing 1 % of bovine serum albumin was added, and the mixtures were allowed to stand at 4°C overnight. After the monoclonal antibody was washed, the supernatant of the faecal solution was added in an amount of 100 μl per well, and incubated at 4°C overnight. After washing, the rabbit DAF polyclonal antibody IgG having a concentration of 4 μg/ml was added in an amount of 100 μl per well, and after the mixtures were allowed to react at room temperature for 2 hours, the wells were washed. Then, a peroxidase-labeled goat F(ab)'2 anti-rabbit IgG (supplied by TAGO, U.S.A) was added in an amount of 100 μl per well, and after the mixtures were allowed to react at room temperature for 2 hours, the reaction products were washed and color-developed with 2,2'-azino-di-3-ethylbenzothiazoline-6-sulfonic acid. The resultant reaction products were measured for absorbance at 415 nm with an automatic ELISA plate reader.

Preparation of calibration curve

[0036] The purified DAF in an amount of 0.1 ng to 10 ng per well was added and allowed to react to prepare a calibration curve. The amount of DAF in samples were calculated on the basis of the calibration curve and expressed

as an amount in 1 g of faeces.

Method of detecting occult blood in faeces

[0037] Guaiac method: Examined with a faecal occult blood slide supplied by Shionogi & Co., Ltd.

[0038] Immunological method: Examined with an OC-Hemodia-Eiken (Trademark) supplied by Eiken Chemical Co., Ltd., Japan.

[0039] The method of detecting DAF molecules in faeces, provided by the present invention, was studied for its usefulness on the basis of the above Method of measuring DAF in faeces and the above Method of detecting occult blood in faeces with regard to 29 patients having colorectal cancer and Control group of 20 people having no colorectal cancer. Figs. 1 and 2 and Tables 1 to 4 show the results.

[0040] Fig. 1 shows concentrations of DAF molecules in faeces from 29 patients having colorectal cancer and Control group of 20 people having no colorectal cancer. In Control group, the concentrations of DAF molecules in faeces from 15 people were below the detectable amount, and the concentrations of DAF molecules in feaces from 5 people were 1 ng in 1 g of faeces, or less. On the other hand, in the 29 patients having colorectal cancer, the concentrations of DAF molecules in faeces from 15 patients were 1 ng in 1 g of feces, or more, the concentrations in faeces from 2 patients were 0.4 to 1 ng, and these concentrations in faeces from 12 patients were below the detectable amount. It is seen that the concentrations of DAF molecules in faeces from colorectal cancer patients and those in faeces from Control group have a significant difference ($p = 0.006$). Further, when the 1 ng/g of DAF molecules in faeces is taken as a boundary, a group of colorectal cancer patients and Control group can be discriminated. When patients showing a concentration of at least 1 ng/g of DAF molecules in faeces are taken as positive, the positive ratio of colorectal cancer patients was 52 % (15 patients/29 patients), or showed a significant difference over 0 % of Control group ($p = 0.0001$).

[0041] Fig. 2 shows differences between concentrations of DAF molecules in faeces before operation of colorectal cancer and concentrations of DAF molecules in faeces after the operation of the colorectal cancer with regard to 5 colorectal cancer patients who preoperatively showed the elevated level of DAF molecules in faeces. The concentrations of DAF molecules in faeces from four patients out of the five patents decreased to be below the detectable sensitivity, and that in faeces from one patient decreased from 8.6 ng/g to 1.4 ng/g.

[0042] Table 1 shows the result of studies of the correlation between the ratio of detection of DAF molecules in faeces and the size of colorectal cancer.

Table 1

		≤ 2 cm*	2 - 5 cm	> 5 cm
DAF	+	3	7	5
	-	2	10	2
Total		5	17	7

Notes: + shows that the concentration of DAF molecules was at least 1 ng/g
- shows that the concentration of DAF molecules was lower than 1 ng/g.

* size of tumor

[0043] DAF molecules were detected in faeces without having anything to do with colorectal cancer sizes.

[0044] Table 2 shows the result of studies of the correlation between the ratio of detection of DAF molecules in faeces and colorectal cancer sites.

Table 2

		Rectum	Rectosigmoid and descending colon	Ascending colon and cecum
DAF	+	4	7	4
	-	3	7	4
Total		7	14	8

Notes: + and - have the same meanings as those in notes to Table 1.

[0045] DAF molecules were detected in faeces without having anything to do with colorectal cancer sites.

[0046] Table 3 shows the result of studies of the correlation between the ratio of detection of DAF molecules in faeces and the TNM stage.

Table 3

		I*	II	III	IV
DAF	+	3	6	4	2
	-	1	6	5	2
Total		4	12	9	4

Notes: + and - have the same meanings as those in notes to Table 1.

* TNM stage of colorectal cancer

[0047] DAF molecules were detected in faeces without having anything to do with TNM stage of colorectal cancer.

[0048] Table 4 shows the result of studies of the correlation between the ratio of detection of DAF molecules in faeces and fecal occult blood testing.

Table 4

		DAF		
		+	-	Total
Guaiac test	+	10	6	16
	-	5	8	13
Immunological test	+	11	12	23
	-	4	2	6

[0049] In 29 patients having colorectal cancer, 16 patients (55 %) were positive in the method of detection of occult blood in faeces according to Guaiac method, and 23 patients (79 %) were positive in the method of detection of occult blood in faeces according to the immunological method. Further, DAF molecules in faeces were detected in 5 patients out of 13 patients for whom the detection according to Guaiac method was negative and in 4 patients out of 6 patients for whom the detection according to the immunological method was negative. In Control group, two people showed that the faecal occult blood testing was positive while the detection of DAF molecules in faeces was negative.

[0050] According to the method of detecting DAF molecules in faeces, provided by the present invention, there is provided a method which enables the detection of DAF in faeces from colorectal cancer patients, which shows a significant difference over the detection of DAF in faeces from Control group of people having no colorectal cancer. Further, according to the method of the present invention, there is provided a method which enables the detection of DAF in faeces from colorectal cancer patients for whom the detection of occult blood in faeces is negative.

Claims

1. A method for detecting colorectal cancer in a patient, which method comprises:

   - reacting an anti-DAF (decay accelerating factor) antibody with a supernatant of a solution of faeces from the patient;
   - measuring an amount of the antibody which has bonded to DAF molecules by an antigen-antibody reaction with the DAF molecules in the supernatant; and
   - detecting thereby if the patient has colorectal cancer

2. A method according to claim 1, wherein the anti-DAF antibody is an anti-DAF monoclonal antibody or anti-DAF polyclonal antibody.

3. A method according to claims 1 to 2, wherein the anti-DAF antibody is converted to a solid phase and then reacted with the supernatant of a faecal solution.

4. A method according to claim 3, wherein the solid phase anti-DAF antibody is reacted with the supernatant of a faecal solution and then the resultant reaction product is reacted with a labelled anti-DAF antibody or fragment thereof to measure the amount of the bonded DAF molecules.

5. A method according to claims 3 or 4, wherein the solid phase anti-DAF antibody is reacted with the supernatant of a faecal solution and then with an anti-DAF antibody and then the resultant reaction product is reacted with a labelled immunoglobulin or fragment thereof to measure the amount of the bonded second anti-DAF antibodies.

6. A method according to claim 5, wherein the immunoglobulin is a monoclonal immunoglobulin or a polyclonal immunoglobulin.

7. A method according to claims 4 to 6, wherein the label is an enzyme label or a radioactive substance label.

8. Use of an anti-DAF antibody in an in vitro method of detection of colorectal cancer by detecting the presence of DAF molecules in faeces.

Patentansprüche

1. Verfahren zur Diagnose eines Colorektal-Karzinoms bei einem Patienten, wobei das Verfahren die Verfahrensschritte umfaßt:

   Umsetzen eines anti-DAF (Decay Accelerating Factor, Zersetzung beschleunigender Faktor) Antikörpers mit einer überstehenden Flüssigkeit einer Lösung des Stuhls des Patienten,

   Messen einer Menge des Antikörpers, die mittels einer Antigen-Antikörperreaktion mit den DAF-Molekülen in der überstehenden Flüssigkeit an DAF-Moleküle gebunden hat und

   dadurch Bestimmen, ob der Patient ein Colorektal-Karzinom hat.

2. Verfahren gemäß Anspruch 1, wobei der anti-DAF-Antikörper ein monoklonaler anti-DAF-Antikörper ist oder ein polyclonaler anti-DAF-Antikörper.

3. Verfahren gemäß Anspruch 1 oder 2, wobei der anti-DAF-Antikörper auf eine Festphase überführt und nachfolgend mit der überstehenden Flüssigkeit einer Stuhllösung umgesetzt wird.

4. Verfahren gemäß Anspruch 3, wobei der Festphasen-anti-DAF-Antikörper mit der überstehenden Flüssigkeit einer Stuhllösung umgesetzt und nachfolgend das resultierende Reaktionsprodukt mit einem markierten anti-DAF-Antikörper oder einem Fragment davon zur Messung der Menge der gebundenen DAF-Moleküle umgesetzt wird.

5. Verfahren gemäß Anspruch 3 oder 4, wobei der Festphasen-anti-DAF-Antikörper mit der überstehenden Flüssigkeit einer Stuhllösung umgesetzt wird und nachfolgend mit einem anti-DAF-Antikörper und das resultierende Reaktionsprodukt dann mit einem markierten Immunoglobulin oder einem Fragment davon zur Messung der Menge des gebundenen zweiten DAF-Antikörper umgesetzt wird.

6. Verfahren gemäß Anspruch 5, wobei das Immunoglobulin ein monoklonales Immunoglobulin ist oder ein polyklonales Immunoglobulin.

7. Verfahren gemäß einem der Ansprüche 4 bis 6, wobei der Marker ein Enzymmarker oder ein Marker einer radioaktiven Substanz ist.

8. Verwendung eines anti-DAF-Antikörpers in einem in vitro-Verfahren zur Bestimmung eines Colorektal-Karzinoms durch Bestimmung der Gegenwart von DAF-Molekülen in Stuhlproben.

Revendications

1. Méthode de détection d'un cancer colo-rectal chez un patient, cette méthode comprenant les étapes consistant :

   - à faire réagir un anticorps anti-DAF facteur d'accélération d'inactivation (« decay ») avec un surnageant d'une solution de fèces du patient ;
   - à mesurer une quantité de l'anticorps qui s'est lié aux molécules de DAF par une réaction antigène-anticorps

avec les molécules de DAF dans le surnageant ; et
- à détecter ainsi si le patient a un cancer colo-rectal.

2. Méthode selon la revendication 1, dans laquelle l'anticorps anti-DAF est un anticorps monoclonal anti-DAF ou un anticorps polyclonal anti-DAF.

3. Méthode selon les revendications 1 à 2, dans laquelle l'anticorps anti-DAF est converti en une phase solide, et on le fait ensuite réagir avec le surnageant d'une solution fécale.

4. Méthode selon la revendication 3, dans laquelle on fait réagir l'anticorps anti-DAF en phase solide avec le surnageant d'une solution fécale, puis on fait ensuite réagir le produit de réaction résultant avec un anticorps anti-DAF ou l'un de ses fragments marqué pour mesurer la quantité des molécules de DAF liées.

5. Méthode selon la revendication 3 ou 4, dans laquelle on fait réagir l'anticorps anti-DAF en phase solide avec le surnageant d'une solution fécale et ensuite avec un anticorps anti-DAF, puis on fait réagir le produit de réaction résultant avec une immunoglobuline ou l'un de ses fragments marqué pour mesurer la quantité des seconds anticorps anti-DAF liés.

6. Méthode selon la revendication 5, dans laquelle l'immunoglobuline est une immunoglobuline monoclonale ou une immunoglobuline polyclonale.

7. Méthode selon les revendications 4 à 6, dans laquelle le marqueur est un marqueur enzymatique ou un marqueur qui est une substance radioactive.

8. Utilisation d'un anticorps anti-DAF dans une méthode *in vitro* de détection de cancer colo-rectal par détection de la présence de molécules de DAF dans les fèces.

Figure 1

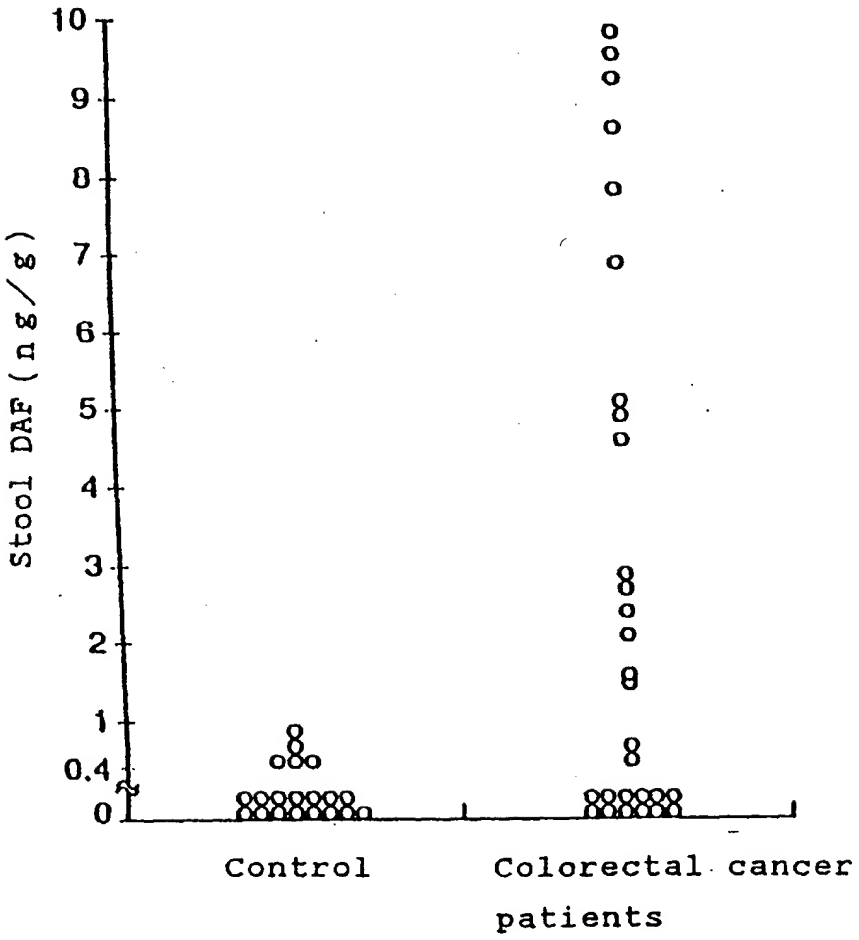
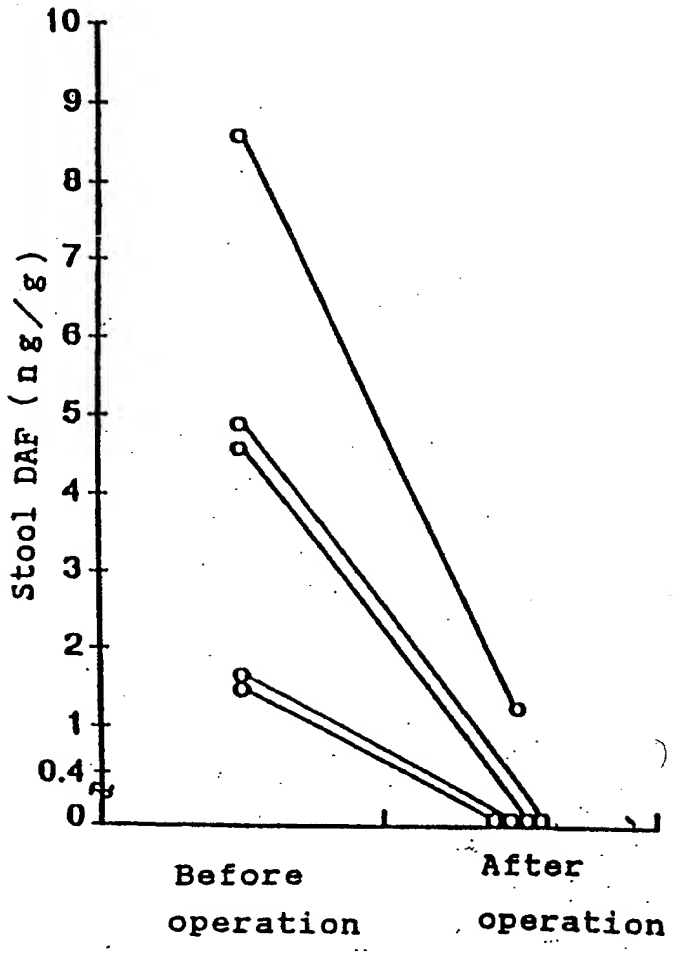

10
9
8
7
6
5
4
3
2
1
0.4
0
Stool DAF (ng/g)
Control
Colorectal cancer patients

Figure 2


10
9
8
7
6
5
4
3
2
1
0.4
0
Stool DAF (ng/g)
Before operation
After operation

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(54) Method of detecting DAF molecules in feces.

(57) A method for detecting the presence of DAF molecules, which are synthesized by colorectal cancer cells, in faeces, the method comprising reacting an anti-DAF antibody with a supernatant of a faecal solution and measuring an amount of antibody bonded to DAF molecules by an antigen-antibody reaction with the DAF molecules in the supernatant. The method is useful in the diagnosis of colorectal cancer.

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Field of the Invention

The present invention relates to a method of detecting DAF molecules in feces. More specifically, it relates to a method of detecting DAF molecules in feces, useful for detecting colorectal cancer by examining the concentration of DAF molecules in feces.

Prior Art of the Invention

In general medical examination, it is conventional practice to detect occult blood in feces for detecting colorectal cancer. When occult blood is detected in feces, the photographic examination based a contrast medium used in the large intestine and the colorectal endoscopic examination of the colon are carried out to detect colorectal cancer. However, occult blood is not always found in feces from the colorectal cancer patients. For this reason, when the examination is based on the detection of occult blood in feces alone, colorectal cancer is sometimes not detected.

It has been recently immunologically found that the expression of DAF molecules is enhanced in a colorectal cancer tissue. DAF molecules, i.e., decay accelerating factor, are glycoproteins which regulate the activation of the autologous complement cascade by promoting the catabolism of C3 and C5 convertases. It has been revealed that almost no DAF molecules are expressed in a normal epithelial cell on colorectal mucosa, but that DAF molecules are intensely expressed, particularly, on an apical surface of a cancer gland duct of a colorectal cancer cell.

The present inventors have assumed that feces from colorectal cancer patients show an increased amount of DAF molecules.

Summary of the invention

It is an object of the present invention to provide a method of easily detecting the occurrence of colorectal cancer.

It is another object of the present invention to provide a method of detecting colorectal cancer by measuring DAF molecules which are synthesized by colorectal cancer cells and present in feces.

It is further another object of the present invention to provide a method of detecting colorectal cancer in the absence of occult blood, by measuring DAF molecules which are synthesized by colorectal cancer cells and present in feces.

According to the present invention, there is provided a method of detecting DAF molecules in feces, which comprises reacting an anti-DAF antibody with a supernatant of a fecal solution and measuring an amount of the antibody which has bonded to DAF molecules by an antigen-antibody reaction with the DAF molecules in the supernatant.

Brief Description of Drawings

Fig. 1 is a graph showing DAF concentrations in feces from colorectal cancer patients and a control group of patients having no colorectal cancer.

Fig. 2 is a graph showing changes of DAF concentrations in feces from pre-operation of colorectal cancer to post-operation.

Detailed Description of the Invention

The detection method of the present invention will be explained in detail hereinafter.

Preparation of supernatant of fecal solution

A predetermined amount of feces are mixed with a buffer solution, the mixture is stirred, and then a supernatant is separated by centrifugation.

Preparation of anti-DAF antibody

Human DAF molecules are purified from erythrocyte membrane to prepare anti-DAF monoclonal antibody and anti-DAF polyclonal antibody.

Measurement of DAF molecules in feces

1) The anti-DAF monoclonal antibody or the anti-DAF polyclonal antibody is converted to a solid phase on a microtiter plate or beads and reacted with the supernatant of feces.

2) After the above reaction product is washed, an enzyme- or radioactive substance-labeled anti-DAF monoclonal antibody or anti-DAF polyclonal antibody is reacted with the washed reaction product to measure an amount of the bonded DAF molecules.

Otherwise,

2') After the above reaction product is washed, an anti-DAF monoclonal antibody or an anti-DAF polyclonal antibody is reacted with the reaction product. The resultant reaction product is washed, and then reacted with an enzyme- or radioactive substance-labeled antimonoclonal immunoglobulin antibody or antipolyclonal immunoglobulin antibody to measure the bonded second anti-DAF antibodies.

Preparation of calibration curve

A calibration curve is prepared on the basis of purified DAF in a known amount, and the DAF amount in a sample is calculated on the basis of the calibration curve and expressed as an amount in 1 g of feces.

According to the method of the present invention, an amount of at least 0.4 ng in 1 g of feces can be accurately measured.

DAF in feces from a colorectal cancer patient, detected by the method of the present invention, is assumed to be derived from cancer cells or erythrocyte in blood from colorectal cancer. Since, however, DAF is detected even in feces from a colorectal cancer patient who is negative with regard to occult blood, it is assumed that above detected DAF includes DAF in feces, derived from cancer cells. DAF in feces from a group of patients having no colorectal diseases (to be referred to as "control group" hereinafter) is less than the detectable sensitivity, and it is therefore assumed that normal colorectal mucosa secretes no DAF.

Colorectal cancer screening based on occult blood in feces has a problem of faulse negative results because that colorectal cancer does not cause detectable bleeding in some cases. The method of the present invention is advantageous in that colorectal cancer which causes no detectable bleeding can be sometimes detected by detecting DAF in feces.

The present invention will be explained more in detail hereinafter with reference to Examples, in which "%" stands for "% by weight" unless otherwise specified.

Example and Comparative Example

Preparation of supernatant of fecal solution

Feces in an amount of 1 to 5 g were mixed with a buffer solution in the same amount as that of the feces, and the mixture was stirred. The buffer solution was a sodium phosphate buffer solution containing 1 % of bovine serum albumin, 0.05 % of Tween and 1 mM of phenylmethylsulfonyl fluoride. The stirred mixture was centrifugally separated for 15 minutes at 20,000 g. The supernatant was sampled and freeze-stored at -80°C before the measurement.

Preparation of anti-DAF mouse monoclonal antibody

1) Purification of human DAF molecules

Erythrocyte was separated from 50 ml of human blood, and 400 ml of a 5 mM sodium phosphate buffer solution containing 1 mM of EDTA and 0.1 mM of phenylmethylsulfonyl fluoride was added to the separated erythrocyte. The mixture was stirred and centrifugally separated at 48,000 g, and a precipitate of an erythrocyte substrate was collected.

The above erythrocyte substrate in an amount of 1 g was diluted to 5 mg/ml with a 5 mM sodium phosphate buffer solution, and 80 ml of butanol was added. The mixture was stirred and then centrifugally separated at 12,000 g, and an aqueous layer between an upper layer of butanol and a lower layer of a precipitate was recovered. Then, the aqueous layer was subjected to a DEAE-Sephacel ion exchange column, and a crude fraction of DAF molecules was separated with a 0.02 M tris buffer solution containing 0.04 M of sodium hydrochloride and 0.1 % of Nonidet P-40 (NP-40).

Further, the crude fraction of DAF was subjected to a phenyl-Sepharose column, and the DAF was separated with a 0.04 M sodium phosphate buffer solution containing 0.3 M of sodium hydrochloride and 0.1 % of

NP-40 and then with a 0.05 M sodium phosphate buffer solution containing 0.05 M of sodium hydrochloride and 1 % of NP-40. Further, the DAF was separated through a hydroxyapatite column.

2) Preparation of anti-DAF mouse monoclonal antibody

A BALB/C mouse was immunized with a suspension of 5 μg of the purified DAF in a Freund adjuvant containing dead tuberculosis bacillus twice at an interval of one week. After three weeks, spleen cells were taken out and fused with NS-1 mouse myeloma cells using polyethylene glycol. The supernatant of a culture of the so-prepared hybridoma was reacted with a nitrocellulose membrane blotted with the purified DAF, and screened by detecting bonded mouse monoclonal antibody with an ABC kit supplied by Vector Laboratories, to give hybridomas which produced four kinds of anti-DAF monoclonal antibodies.

All of the produced monoclonal antibodies were IgG1 kappa. Of these four kinds of monoclonal antibodies, 1C6 monoclonal antibody which recognized an active portion of DAF molecules was used for the detection of stool DAF. Hybridoma which produced 1C6 monoclonal antibody was intraperitoneally injected into a mouse to prepare ascites, which was sampled.

1C6 anti-DAF monoclonal antibody IgG was separated and purified from the ascites by an ammonium sulfate precipitation method and DEAE chromatography (using "Toyopearl" supplied by Tosoh Corp.).

Preparation of rabbit anti-DAF polyclonal antibody

The purified DAF was infused into 10 ml of rabbit erythrocyte by incubating it at 37°C for 1 hour. This erythrocyte was washed and dissolved, and cytoplasmic membrane was centrifugally separated. The cytoplasmic membrane was suspended in a Freund adjuvant containing dead tuberculosis bacillus, and the rabbit from which the erythrocyte had been separated was immunized with the so-prepared suspension. Blood was taken from the immunized rabbit, serum was separated, and rabbit IgG was separated and purified by an ammonium sulfate precipitation method and DEAE chromatography.

The specificity of the above-obtained antibody was examined by an immunoblotting method. That is, a crude extract of human erythrocyte substrate was electrophoresed in SDS polyacrylamide gel, blotted into a nitrocellulose membrane, and reacted with the rabbit DAF polyclonal antibody. When bonded rabbit IgG antibody was detected with a Vectastain ABC kit (Vector Laboratories, Inc., CA), a single band was detected in a site corresponding to the molecular weight of 70 kD of the DAF molecules, so that the specificity of the rabbit anti-polyclonal antibody was confirmed.

Measurement of DAF molecules in feces

The 1C6 DAF mouse monoclonal antibody having a concentration of 2 μg/ml was placed in wells of a microtiter plate in an amount of 100 μl per well. After a reaction at 4°C for 12 hours, a sodium phosphate buffer solution containing 1 % of bovine serum albumin was added, and the mixtures were allowed to stand at 4°C overnight. After the monoclonal antibody was washed, the supernatant of the fecal solution was added in an amount of 100 μl per well, and incubated at 4°C overnight. After washing, the rabbit DAF polyclonal antibody IgG having a concentration of 4 μg/ml was added in an amount of 100 μl per well, and after the mixtures were allowed to react at room temperature for 2 hours, the wells were washed. Then, a peroxidase-labeled goat F(ab)'2 anti-rabbit IgG (supplied by TAGO, U.S.A) was added in an amount of 100 μl per well, and after the mixtures were allowed to react at room temperature for 2 hours, the reaction products were washed and color-developed with 2,2'-azino-di-3-ethylbenzothiazoline-6-sulfonic acid. The resultant reaction products were measured for absorbance at 415 nm with an automatic ELISA plate reader.

Preparation of calibration curve

The purified DAF in an amount of 0.1 ng to 10 ng per well was added and allowed to react to prepare a calibration curve. The amount of DAF in samples were calculated on the basis of the calibration curve and expressed as an amount in 1 g of feces.

Method of detecting occult blood in feces

Guaiac method: Examined with a fecal occult blood slide supplied by Shionogi & Co., Ltd.

Immunological method: Examined with an OC-Hemodia-Eiken supplied by Eiken Chemical Co., Ltd., Japan.

The method of detecting DAF molecules in feces, provided by the present invention, was studied for its usefulness on the basis of the above Method of measuring DAF in feces and the above Method of detecting occult blood in feces with regard to 29 patients having colorectal cancer and Control group of 20 people having no colorectal cancer. Figs. 1 and 2 and Tables 1 to 4 show the results.

Fig. 1 shows concentrations of DAF molecules in feces from 29 patients having colorectal cancer and Control group of 20 people having no colorectal cancer. In Control group, the concentrations of DAF molecules in feces from 15 people were below the detectable amount, and the concentrations of DAF molecules in feces from 5 people were 1 ng in 1 g of feces, or less. On the other hand, in the 29 patients having colorectal cancer, the concentrations of DAF molecules in feces from 15 patients were 1 ng in 1 g of feces, or more, the concentrations in feces from 2 patients were 0.4 to 1 ng, and these concentrations in feces from 12 patients were below the detectable amount. It is seen that the concentrations of DAF molecules in feces from colorectal cancer patients and those in feces from Control group have a significant difference ($p = 0.006$). Further, when the 1 ng/g of DAF molecules in feces is taken as a boundary, a group of colorectal cancer patients and Control group can be discriminated. When patients showing a concentration of at least 1 ng/g of DAF molecules in feces are taken as positive, the positive ratio of colorectal cancer patients was 52 % (15 patients/29 patients), or showed a significant difference over 0 % of Control group ($p = 0.0001$).

Fig. 2 shows differences between concentrations of DAF molecules in feces before operation of colorectal cancer and concentrations of DAF molecules in feces after the operation of the colorectal cancer with regard to 5 colorectal cancer patients who preoperatively showed the elevated level of DAF molecules in feces. The concentrations of DAF molecules in feces from four patients out of the five patents decreased to be below the detectable sensitivity, and that in feces from one patient decreased from 8.6 ng/g to 1.4 ng/g.

Table 1 shows the result of studies of the correlation between the ratio of detection of DAF molecules in feces and the size of colorectal cancer.

Table 1

	≤ 2 cm*	2 - 5 cm	> 5 cm
DAF +	3	7	5
-	2	10	2
Total	5	17	7

Notes: + shows that the concentration of DAF molecules was at least 1 ng/g
- shows that the concentration of DAF molecules was lower than 1 ng/g.
* size of tumor

DAF molecules were detected in feces without having anything to do with colorectal cancer sizes.

Table 2 shows the result of studies of the correlation between the ratio of detection of DAF molecules in feces and colorectal cancer sites.

Table 2

	Rectum	Rectosigmoid and descending colon	Ascending colon and cecum
DAF +	4	7	4
-	3	7	4
Total	7	14	8

Notes: + and - have the same meanings as those in notes to Table 1.

DAF molecules were detected in feces without having anything to do with colorectal cancer sites.

Table 3 shows the result of studies of the correlation between the ratio of detection of DAF molecules in feces and the TNM stage.

Table 3

	I*	II	III	IV
DAF +	3	6	4	2
-	1	6	5	2
Total	4	12	9	4

Notes: + and - have the same meanings as those in notes to Table 1.
* TNM stage of colorectal cancer

DAF molecules were detected in feces without having anything to do with TNM stage of colorectal cancer.

Table 4 shows the result of studies of the correlation between the ratio of detection of DAF molecules in feces and fecal occult blood testing.

Table 4

		DAF		
		+	-	Total
Guaiac test	+	10	6	16
	-	5	8	13
Immunological test	+	11	12	23
	-	4	2	6

In 29 patients having colorectal cancer, 16 patients (55 %) were positive in the method of detection of occult blood in feces according to Guaiac method, and 23 patients (79 %) were positive in the method of detection of occult blood in feces according to the immunological method. Further, DAF molecules in feces were detected in 5 patients out of 13 patients for whom the detection according to Guaiac method was negative and in 4 patients out of 6 patients for whom the detection according to the immunological method was negative. In Control group, two people showed that the fecal occult blood testing was positive while the detection of DAF molecules in feces was negative.

According to the method of detecting DAF molecules in feces, provided by the present invention, there is provided a method which enables the detection of DAF in feces from colorectal cancer patients, which shows a significant difference over the detection of DAF in feces from Control group of people having no colorectal cancer. Further, according to the method of the present invention, there is provided a method which enables the detection of DAF in feces from colorectal cancer patients for whom the detection of occult blood in feces is negative.

Claims

1. A method for detecting the presence of decay accelerating factor (DAF) molecules in faeces, which comprises reacting an anti-DAF antibody with a supernatant of a faecal solution and measuring an amount of the antibody which has bonded to DAF molecules by an antigen-antibody reaction with the DAF molecules in the supernatant.

2. A method according to claim 1, wherein the anti-DAF antibody is an anti-DAF monoclonal antibody or anti-

DAF polyclonal antibody.

3. A method according to claims 1 to 2, wherein the anti-DAF antibody is converted to a solid phase and then reacted with the supernatant of a faecal solution.

4. A method according to claim 3, wherein the solid phase anti-DAF antibody is reacted with the supernatant of a faecal solution and then the resultant reaction product is reacted with a labelled anti-DAF antibody or fragment thereof to measure the amount of the bonded DAF molecules.

5. A method according to claims 3 or 4, wherein the solid phase anti-DAF antibody is reacted with the supernatant of a faecal solution and then with an anti-DAF antibody and then the resultant reaction product is reacted with a labelled immunoglobulin or fragment thereof to measure the amount of the bonded second anti-DAF antibodies.

6. A method according to claim 5, wherein the immunoglobulin is a monoclonal immunoglobulin or a polyclonal immunoglobulin.

7. A method according to claims 4 to 6, wherein the label is an enzyme label or a radioactive substance label.

8. A method for diagnosis of colorectal cancer, which comprises detecting the presence of DAF molecules in faeces by a method according to any preceding claim.

9. An anti-DAF antibody for use in a method of detection of colorectal cancer by detecting the presence of DAF molecules in faeces.

Figure 1

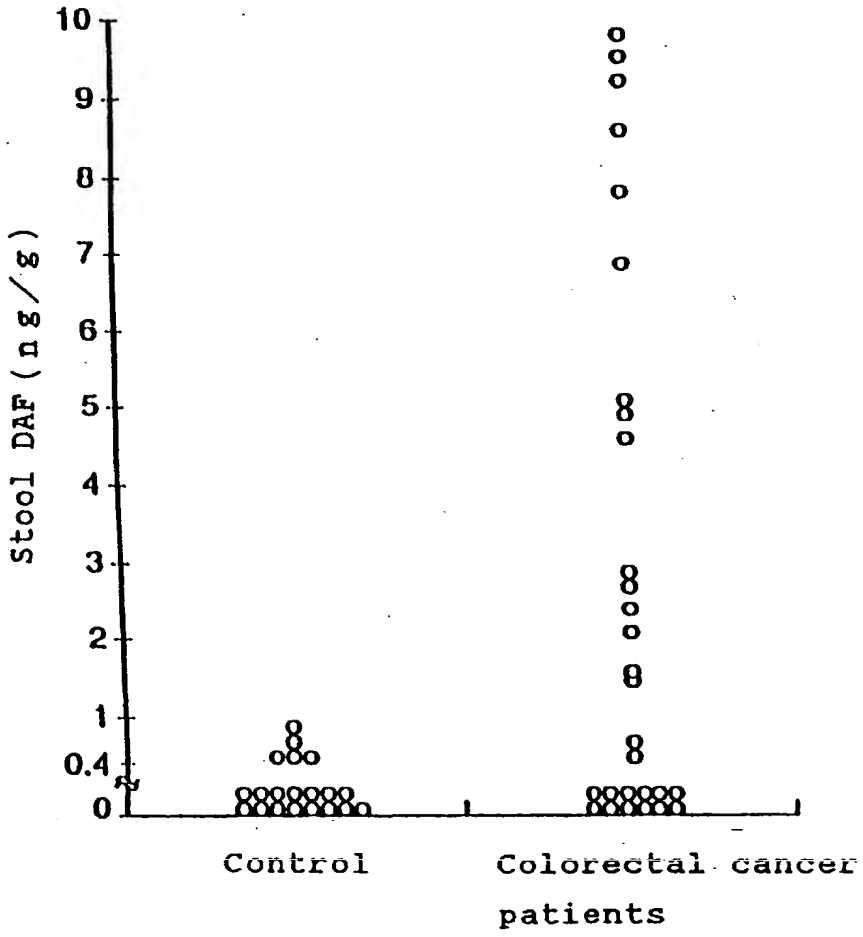
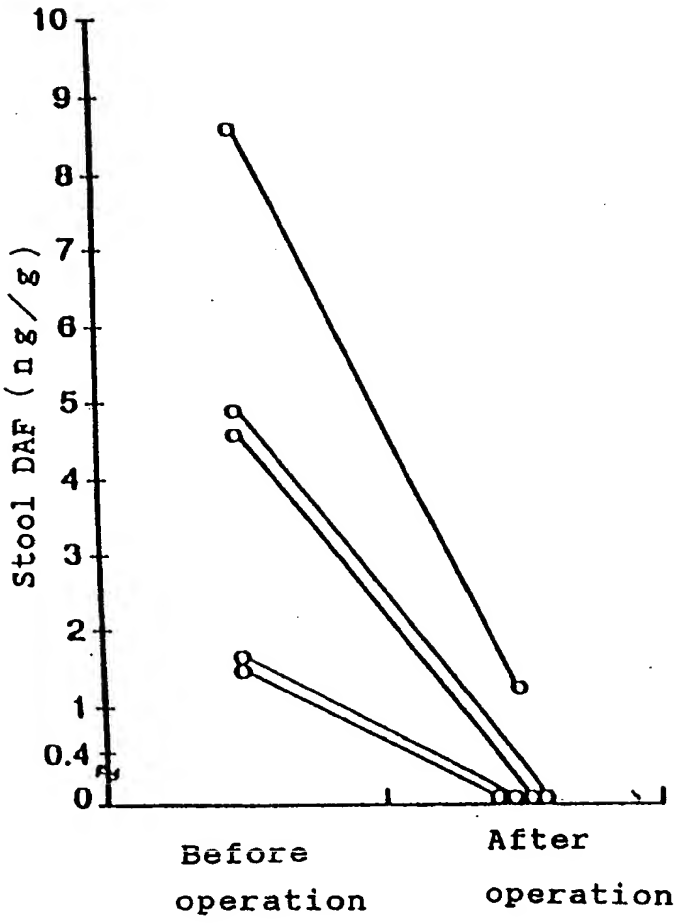

Stool DAF (ng/g)
10
9
8
7
6
5
4
3
2
1
0.4
0
Control
Colorectal cancer patients

Figure 2


10
9
8
7
6
5
4
3
2
1
0.4
0
Stool DAF (ng/g)
Before operation
After operation

European Patent Office

EUROPEAN SEARCH REPORT

Application Number EP 95303753.8

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 6)
X,P	JP - A - 06 317 588 (YAMANOUCHI PHARMACEUT CO. LTD.) * Totality, especially abstract * --	1,2	G 01 N 33/53 G 01 N 33/574 G 01 N 33/577
X	CHEMICAL ABSTRACTS, vol. 120, no. 15, April 11, 1994 Columbus, Ohio, USA T. HARA et al. "A monoclonal antibody against human decay-accelerating factor (DAF, CD55), D17, which lacks reactivity with semen-DAF", page 807, no. 189 246v; & Immunol. Lett. 1993, 37(2-3), 145-52. --	1,2	
X	CHEMICAL ABSTRACTS, vol. 106, no. 23, June 8, 1987 Columbus, Ohio, USA M.A. DAVITZ et al. "Isolation of decay accelerating factor (DAF) by a two-step procedure and determination of its N-terminal sequence" page 552, no. 194 206x; & Immunol. Methods 1987, 97(1), 71-6 --	1,2	TECHNICAL FIELDS SEARCHED (Int. Cl. 6) G 01 N 33/00
A	WO - A - 86/07 062 (NEW YORK UNIVERSITY) * Abstract; claims * ----	1,2	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
VIENNA	23-08-1995	SCHNASS

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
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T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
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